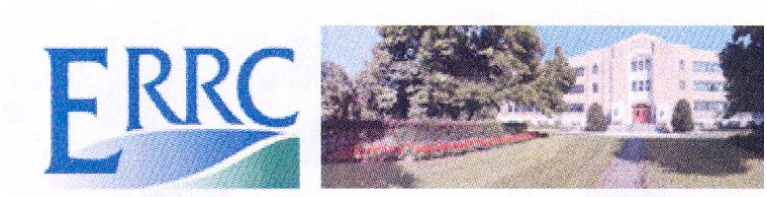

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Title: *Cyclospora cayetanensis:* Research Methodology and Control by Irradiation

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Citation: In "Use of Irradiation to Ensure Hygienic Quality of Fresh, Pre-Cut and Vegetables and Other Minimally Processed Food of Plant Origin" Report of First FAO/IAEA Meeting 5-9, November 2001, Rio De Janeiro, 7 pp. (2001) 1-7

Number: 7439

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Cyclospora cayetanensis: Research methodology and control by irradiation

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2 ## *Cyclopsora cayetanensis*: introduction and epidemiology

- Coccidian parasite; spherical oocysts ~9 nm in diameter
- Reports of recovery of *C. cayetanensis* from Old World primates, but humans may be the only true hosts
- Disease progression:
  - ingestion of a sporulated oocyst containing 4 sporozites
  - sporozoites penetrate epithelial lining of intestine
  - increasing damage to host cells concurrent with parasite's asexual division, mobility and sexual reproduction
  - new oocysts sloughed into feces, along with damaged epithelium

3

4 ## *C. cayetanensis*: introduction and epidemiology

- Clinical symptoms
  - signs start 1-7 days post-ingestion of oocysts
  - mild infections may produce little or no outward signs
  - more extensive infections may show prolonged watery diarrhea, abdominal cramping, weight loss, anorexia, vomiting and/or fever
- Extensive treatment with sulfa-type drugs can be effective

5 ## *C. cayetanensis*: introduction and epidemiology

- Infection historically associated with poor sanitation
  - pediatric gastroenteritis in developing nations

- adult infection in travelers from industrialized nations visiting loci of infection

- 1996: 1465 cases in USA, Canada associated with Guatemalan raspberries (Herwaldt and Ackers, N Engl J Med 1997 May 29;336(22):1548-56)
- April-June 1997: 1012 cases in USA, Canada, Guatemalan raspberries again implicated (Herwaldt and Beach, Ann Intern Med 1999 Feb 2;130(3):210-20)
- May 1997: Guatemala voluntarily suspends export of fresh raspberries, outbreak ends

6 ☐ ## *C. cayetanensis*: introduction and epidemiology

- Outbreaks in North America occur more frequently in spring, early summer
- Relation to time of peak importation of fresh fruits and vegetables suggested
- Long distance transport of commodities, multiple handling stations increase possibility of contamination between farm and table
- Washing foods before consumption is a key step, but it does not remove 100% of oocysts (Ortega, Y.R. et al. 1997 Am. J. Trop. Med. Hyg. 57: 683-686)

7 ☐ ## *C. cayetanensis*: detection methodology

- US-FDA BAM: Concentration and Preparation of Cyclospora from Berries for the Polymerase Chain Reaction (PCR) and Microscopy (vm.cfsan.fda.gov/~ebam/bam-19a.html)
- Epi-illuminated Fluorescence Microscope; UV 1A filter block (Excitation Filter, EX 365/10; Dichroic mirror, DM 400; Barrier Filter, BA-400; or equivalent)
- PCR: buffers, reagents, thermocycler, gels, film (protocols should be validated for each material/isolate)

8 ☐

- Wash method for fresh produce (berries, lettuce, etc.) or puree
- Stomacher bag, 250 ml of deionized water
- 250-500 g of produce
  - berries +/- 1 berry; USE ONLY INTACT BERRIES
  - juice from cut or broken berries may be inhibitory to PCR
  - debris may interfere with microscopy
- 250 g of puree (juice and debris are unavoidable)

9 ☐

- Invert and agitate gently. Avoid damaging produce. Centrifuge 1500 X g for 10 minutes
- Decant supernatant, retaining 1 ml of supernatant and to resuspend pellet fraction.
- Appropriate aliquots of resuspended pellet will be removed from this tube for microscopy and PCR.
- Store the remaining portion at 4°C for up to one month. After one month, dilute the remaining material with an equal volume of 2.5% potassium dichromate, mix, and store at 4°C.

10 ☐

- Prepare wet mount of 10 µl sediment.
- View under UV light at 400 X. Cyclospora oocysts fluoresce cobalt blue with UV-1A filter, blue-green with broader UV illumination.
- Confirm cyst size of 8-10 µm with microscope reticle, compare presumptive oocysts to those in a known standard.
- Switch from epi-fluorescence microscopy to bright field or DIC to confirm internal structures
- Seal slides, document positive samples with photographs taken at multiple planes.

11 ☐

- 100 µl of produce sediment. Thaw, microcentrifuge. 14,000 RPM (15,800 X g) for 3 min and discard supernatant.
- Wash pellet once with 100 µl TE buffer, centrifuge at 14,000 RPM for 3 min. Discard supernatant and resuspend in 100 µl TE
- Complete 3 freeze/thaw cycles, each 2 min in liquid nitrogen or a dry ice-ethanol bath followed by 2 min in a 98°C water bath. Add 0.1 ± 0.02 g glass beads to extract.

12 ☐

- Vortex for 5 min, chill on ice for 5 min.
- Centrifuge sample extract at 14,000 RPM (15,800 X g) for 3 min. Transfer supernatant to new sterile microcentrifuge tube.
- This extract can be stored frozen (-20°C) until ready for the PCR analysis, or if needed as reserve in case of template inhibition problems.
- Combine 20 µl sample extract and 2 µl freshly prepared Non-fat Milk Solution in 1 ml sterile deionized water. The entire 22 µl will be used as template in a 100 µl PCR amplification.

13 ☐

- PCR primers: CYCF1E, CYCR2B, CYCF3E and CYCR4B (Relman et. al., J. Infect. Dis.173:440-445, 1996)

14 ☐

- Two sequential PCR runs
- #1 uses CYCF1E and CYCR2B primers, probing the template DNA extracted from the sample
- #2 uses CYCF3E and CYCR4B primers, probing the template DNA from Step #1
- Agarose/ethidium bromide gel electrophoresis, UV transillumination. Predicted size of DNA marker after F1E/R2B, is 651 bp; after F3E/R4B is 308 bp.

★Amplified product after the first round may not be visible; only product from the second round of PCR should be electrophoresed.

15 ☐

- PCR product of 308 bp after the second PCR round a presumptive positive for Cyclospora or Eimeria.

- Digestion of the amplification product with MnlI distinguishes Cyclospora from Eimeria
- Prepare separate restriction digests
  - each presumptive positive PCR amplification product
  - amplification products from control *Cyclospora cayetanensis* and *Eimeria tenella* strains.
- Agarose/ethidium bromide
  gel electrophoresis

16

- Microscopy is a fundamental tool for detection
- PCR will reliably indicate the presence of *C. cayetanensis*, and can discriminate between Cyclospora and Eimeria
- For both methods, comparison with known standards is essential

★PCR will amplify DNA from dead cells - not a diagnostic tool to evaluate post-irradiation survival or infectivity. Only indicates presence/absence.

17 ## *C. cayetanensis*: ionizing radiation

- Multiple studies have been completed on *C. cayetanensis* (Dubey, Thayer, Speer, and Shen, 1998)
- Typically trials were conducted with 50,000 sporulated or 50,000 unsporulated oocysts
- Excystation occurred at doses through 0.5 kGy
- Sporulation occurred at doses up to 1.0 kGy though there was a dose dependent delay, morphological changes, and reduction in sporulation

18 ## *C. cayetanensis*: treatment validation

- No animal model exists; difficult to validate inactivation
- Molecular techniques may provide some additional tools, but validation of inactivation treatments requires human experimentation
- Severity of symptoms, frequency of mortality hinders efforts to recruit volunteers
- A good model pathogen would provide results that would reliably predict the behavior of irradiated *C. cayetanensis*

19 ## *Toxoplasma gondii* oocyst as model for coccidian parasite irradiation

- Low host specificity, infects many mammals, including human. Good animal model (mouse) readily available.
- Infection generally asymptomatic in adults; low risk of hepatitis, blindness, spontaneous abortion
- Transmitted by fecal, oral and meat
- Very efficient bioassay — 1 oocyst can be fatal in test subject.
- Mortality in mice correlated with dose exposure

20 ## *T. gondii*: methodology

- Dubey, Jenkins, Thayer 1996. J. Parasitol. 82:724-727
- Oocysts obtained by sugar flotation of feces from cats fed tissues cysts of VEG strain.
- Oocysts sporulated in 2% H_2SO_4 at room temperature, 7 days. Stored in H_2SO_4 at 4C until use.
- ~10^6 sporulated oocysts neutralized in 3% NaOH, centrifuged, resuspended in 1 ml 0.9% NaCl
- Gamma irradiation was performed at room temperature
- Treated oocysts were inoculated orally into mice

21 *T. gondii*: methodology

- Mice that died after inoculation were examined for tissue cysts
- Surviving mice were bled periodically to isolate antibodies to *T. gondii*
- All mice were sacrificed after 2 months. A portion of the mouse tissue was fed to 2 *T. gondii*-free cats.
- The cats were bled to isolate antibodies, and sacrificed after 25 days to identify tissue cysts

22 *T. gondii* radiation biology

- Most mice fed non-irradiated oocysts or oocysts irradiated to 0.1 kGy or less died of acute toxoplasmosis
- At doses 0.2 and above, no tissue cysts were found.
- Antibodies to *T. gondii* were found in 11 of 40 mice fed oocysts irradiated to 0.2-0.5 kGy
- Cats fed tissue from seropositive mice did not shed *T. gondii* oocysts in feces, did not develop antibodies to *T. gondii* and did not develop tissue cysts

23 *T. gondii*: strain radiation sensitivity

- Dubey and Thayer, J. Parasitol. 80:764-767 (1994)
- Mice were inoculated with 95 new strains of T. gondii, isolated from pigs. Also inoculated with 10 existing laboratory strains
- Brain tissue vacuum packed, irradiated to 0.0 (control), 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.9 kGy
- Treated samples were bioassayed in mice, cats or both
- Tissue cysts of <u>all strains</u> were rendered nonviable at 0.4 kGy
- 0.25 kGy had identical efficacy when delivered at -4, 0, 4, 8, 12 or 16C. No effect of irradiation temperature.

24 *T. gondii*: oocysts irradiated <u>before</u> sporulation

- Unsporulated oocysts irradiated
  - Exp't 1: 0.0 (control), 0.2, 0.4, 0.6 and 0.8 kGy
  - Exp't 2: 0.0 (control), 3.5 and 4.0 kGy
- Incubated in 2% H_2SO_4 at room temperature for 30 (#1) or 12 (#2) days to allow sporulation
- Microscopy to confirm germination, fed to mice

- Development of cysts evaluated by bioassay

25 *T. gondii*: oocysts irradiated before sporulation

* Antibodies to *T. gondii* not found in these mice. No evidence of disease demonstrated by bioassay

- (Dubey et al. 1998 Int. J Parasitology 28:369-375)

26 *T. gondii*: effect of sporulation stage

* Oocysts were irradiated before sporulation (2 sporocysts formed) and then bioassayed in mice.

** No. of mice *T. gondii* positive of no. of mice inoculated.

27

- Oocysts sporulated before irradiation
- Raspberries either sprayed via atomiser (10^6 oocysts) or injected with suspension (10^4 oocysts)
- Both sets irradiated to 0.4 kGy
- Berries homogenized in saline, pelletized. Sediment filtered (200 and 90 µm screen) and repelletized.
- Sediment fed to mice. Bled for antibody isolation and sacrificial bioassay.

28 *T. gondii*: oocysts irradiated on raspberries

29 *T. gondii*: conclusions

- *T. gondii* sporulates after doses up to 1.0 kGy, but is nonviable and unable to cause disease after doses of 0.4 kGy
- *T. gondii* oocysts irradiated at ≥ 0.4 kGy can excyst but do not multiply in the host
- Sporotozoites from irradiated oocysts can induce antibody formation in the host, even in the absence of histopathological signs
- *In vitro* measurement of sporulation alone insufficient to properly evaluate efficacy of irradiation

30 *T. gondii*: conclusions

- Biology and radiation sensitivity make T. gondii a good model for *C. cayetanensis*
- Requirement for animal model to validate efficacy of irradiation, as *in vitro* observations do not reliably indicate ability to cause disease
- Elimination/inactivation of T. gondii at relatively low doses, ~0.4 kGy
- This dose level is tolerable and beneficial for many fruits and vegetables, including raspberries
- US-FDA regulations: max. of 1.0 kGy for disinfestation, delay of maturation of produce. Proposal to increase limit is under review.

31 Research questions

- Effect of different fruit and/or vegetable substrates on radiation sensitivity of *C. cayetanensis* or *T. gondii*

- Effect of isolate and/or strain variation, in combination with changes in substrate, processing conditions (temperature, MAP, etc.)
- Radiation processing of other coccidians (e.g. Cryptosporidium)
- Further validation and acceptance of *T. gondii* as a model for *C. cayetanensis* by regulatory bodies, and interaction of different regulatory agencies in exporting/importing countries

32 Electronic reference resources

- US-FDA: Concentration and Preparation of Cyclospora from Berries for the Polymerase Chain Reaction (pcr) and Microscopy
- US-CDC: *C. cayetanensis* FAQ, epidemiology links
  (www.cdc.gov/ncidod/dpd/parasites/cyclospora/factsht_cyclospora.htm)
- Kansas State University Parasitology Laboratory
  (www.ksu.edu/parasitology/cyclospora/cyclospora.html)
- USDA: Food Irradiation (overview, including discussion of *C. cayetanensis*)
  (www.ars.usda.gov/is/pr/1997/971210.htm)